Endolysosomal Processing of Exogenous Antigen into Major Histocompatibility Complex Class I-Binding Peptides

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Abstract

An alternative endolysosomal pathway has recently been suggested for the processing of MHC-I-binding peptides, and peptide/MHC-I complexes have been demonstrated in this compartment. However, it remains unclear where in the antigen-presenting cells such peptides are processed, in the endolysosomes themselves or in the proteasomal complex. Here, we have investigated this using monoclonal antibodies specific for the immunodominant SIINFEKL/Kb complex (25-D1) or for the carbohydrate part of Db- or Kb-binding glycopeptides in combination with inhibitors for classical and endolysosomal MHC-I-processing pathways. Alternative processing was detected in both wt and $TAP1^{-/-}$ immature DC (iDC) as the expression of SIINFEKL/Kb complexes on the surface of OVA-treated cells in the presence of Brefeldin A (BFA) or lactacystin and their absence in the presence of the lysosomotropic amines ammonium chloride, chloroquine and methylamine. Internalized Db- and Kb-binding glycopeptides, detected with high specificity using an anti-galabiose (Gal2) monoclonal antibody, were found to appear on the cell surface of BFA-treated cells after intracellular MHC-I-binding. Peptide exchange in Kb was demonstrated as the gradual appearance of SIINFEKL/Kb complexes on BFA-treated cells which earlier had been saturated with another Kb-binding peptide. Our data support the presence of a fully functional endolysosomal processing pathway in iDC guided by the chaperone function of MHC-I molecules.

Introduction

MHC class I-restricted T-cell responses require the presentation of peptides on the surface of dendritic cells (DC) [1]. Intracellular proteins are processed in proteasomes, with preference for defective ribosomal products [2], but more recently also other proteolytic enzymes have been reported to take part in the formation of MHC-I-binding peptides. These include trimming enzymes in the secretory pathway, the oligopeptidase TPPII (tripetidylpeptidase II) as well as other cytosolic proteases [3, 4]. It has been estimated that this nonproteasomal processing of intracellular proteins could contribute to one third of all MHC-I-bound peptides [3].

However, most CTL responses are probably initiated by the uptake of exogenous antigens into DC in a process called 'cross-priming' [5]. Processing of internalized protein antigens can occur either in the classical MHC-I pathway by 'leakage' of these from the endolysosomes into the cytosol [6, 7] or by alternative pathways in endolysosomes or in phagosomes [8–15]. Evidence for endolysosomal processing has been presented for B cells, macrophages and neutrophils [8–13, 16] and more lately also in DC [17–19]. Lizée *et al.* have directly demonstrated peptide/MHC-I complexes in the endolysosomal compartment and in addition described a tyrosine-based-targeting signal in the cytoplasmic domain of MHC-I, required for the routing of these molecules to endolysosomes [17]. Interestingly, they also showed that mutated Kb, lacking the targeting signal, was defect in presenting two immunodominant viral epitopes in transgenic mice. However, from this work, it was not clear where in the DC the Kb-binding peptides were produced. Phagosomal MHC-I processing relies on the export of partially digested antigen to the cytosol, by the Sec61 complex, peptide formation in the proteasome complex and TAP-mediated transport of these peptides back into phagosomes. Thus, it has been proposed that phagosomes constitute a complete processing system, distinct from the classical MHC-I pathway [20].

Exhibit 3

The different MHC-I-processing pathways can be distinguished using inhibitors for the Golgi system, for proteolysis in proteasomes or for endolysosomal activity like Brefeldin A (BFA), lactacystin and lysomotropic amines, respectively [21–23]. Both BFA and lactacystin block classical processing, whereas the lysosomotropic amines inhibit endolysosomal processing. Recently, using some of these drugs, we have demonstrated the transient expression of an endolysosomal MHC-I-processing pathway in immature DC (iDC) using the T-cell hybridoma B3Z specific for the OVA-derived SIINFEKL/Kb complex [24] as a functional read-out [25].

In the present work, we have collected additional evidence for endolysosomal MHC-I processing in iDC using monoclonal antibodies specific either for the SIINFEKL/Kb complex [26] or for the carbohydrate part of Db- and Kb-binding glycopeptides [27]. We have found that iDC, but not cells with an intermediate phenotype (imDC), can process exogenous OVA into the SIINFEKL/Kb complex and that this most likely occurs by peptide exchange in endolysosomes.

Materials and methods

Mice, cells and reagents. B6 and TAP1$^{-/-}$ B6 [28] mice were bred and housed in the animal facility at the Microbiology and Tumorbiology Center (MTC), Karolinska Institute, Stockholm, Sweden. TAP1$^{-/-}$ mice were backcrossed with B6 mice at least six generations. DC generated from TAP1$^{-/-}$ B6 mice showed highly reduced MHC-I expression, due to the inability to transport endogenous peptide into the ER (endoplasmic reticulum). Mice at 6–9 weeks of age were used for generation of DC *in vitro*. TAP2-deficient RMA-S cells are derived from the Rauscher leukaemia virus-induced T-cell lymphoma RBL-5 [29]. RMA-S cells were maintained in RPMI 1640 medium, supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine (complete medium). OVA (ovalbumin, grade V), BFA (Brefeldin A), lactacystin, ammonium chloride, chloroquine, methylamine and pronase E were purchased from Sigma, Stockholm, Sweden. The ovalbumin peptide 257–264 (SIINFEKL) was synthesized and HPLC-purified by ThermoHybaid, Ulm, Germany. The Db- and Kb-binding glycopeptides ASN9-6h-Gal2 and SII8-4h-Gal2 were synthesized using a solid-phase tBoc system, as earlier described [30]. Monoclonal antibody 25-D1, specific for the SIINFEKL/kb complex [26], was obtained from Dr Ronald Germain (NIH, Bethesda, MD, USA). Monoclonal antibody MC2102, specific for galabiose (Gal2) [27], was obtained from Dr Arne Lundblad, Linköping, Sweden. F(ab′)$_2$ FITC-labelled rabbit antimouse secondary antibody was purchased from Dakopatts, Glostrup, Denmark and F(ab′)$_2$ Alexa Fluor 647-labelled goat antimouse from Molecular Probes, Leiden, The Netherlands.

Generation of DC. DC were cultured from mouse bone marrow, as described [31]. Briefly, 6–9 week-old B6 (H-2b) or TAP1$^{-/-}$ B6 (H-2b) female mice were killed and bone marrow flushed from the large bones. Erythrocytes were lysed with ammonium chloride. Lymphocytes and Ia-positive cells were removed with a set of monoclonal antibodies, including GK1.5 (anti-CD4), HO2.2 (anti-CD8), B21-2 (anti-Ia) and RA3-3a/6.1 (anti-B220/CD45R) (hybridomas obtained from American Type Culture Collection, Rockville, MD, USA) and rabbit complement (Calbiochem, La Jolla, CA, USA). The remaining population (6–8 × 10^6 cells/well) was placed in 6-well plates in complete medium, supplemented with rGM-CSF (10 ng/ml) (PeproTech, London, UK) and recombinant interleukin-4 (rIL-4) (10 ng/ml) (Biosite, Stockholm, Sweden). Medium was changed every second day. On the second day, 75% of the medium was aspirated after gently swirling the plates, and then the remaining nonadherent cells, mostly in small, loosely attached aggregates, were transferred to new 6-well plates. By day 5, iDC appeared in large clusters and collected. imDC were derived from dispersed iDC subcultured for additional 2 days in large dishes.

iDC expressed low levels of MHC-I and -II, CD40, costimulatory molecules CD80/CD86 and CD11c as compared to imDC, which expressed higher levels of these markers, as earlier reported [25]. Although not all early iDCs were CD11c positive, they homogenously expressed higher levels of MHC antigens compared to resting or activated macrophages, excluding a contamination of macrophages in the iDC population.

Antibody staining and flow cytometry. Cells (1–2 × 10^6) were suspended in phosphate-buffered saline, incubated with the primary monoclonal antibodies for 30 min on ice, washed and incubated with a F(ab′)$_2$ FITC-labelled rabbit antimouse or F(ab′)$_2$ Alexa Fluor 647-labelled goat antimouse secondary antibody for an additional 30 min. After washing, cells were analysed by flow cytometry using a Becton Dickinson FACScan system. Forward and side scatter gates were set to select the peak of the single viable cells and thus to exclude dead cells and cellular aggregates.

OVA processing and presentation. iDC and imDC from both wt and TAP1$^{-/-}$ mice were collected and incubated either in medium or in medium containing BFA (10 µg/ml), lactacystin (10 µg/ml), ammonium chloride (20 mM), chloroquine (25 µM) or methylamine (5 mM) for 30 min before the addition of OVA protein (10 mg/ml). All the inhibitors were included for the whole processing time of 4 h at 37 °C. Cells were then washed and stained with the 25-D1 monoclonal antibody and then with the F(ab′)$_2$ Alexa Fluor 647-labelled goat antimouse secondary antibody.

Peptide uptake and intracellular Kb binding. Peptide uptake and Kb binding was performed as earlier described [32]. RMA-S cells (precultured at 26 °C overnight) or iDC were incubated with glycopeptides ASN9-6h-Gal2 or SII8-4h-Gal2 (30 µg/ml) for 2 h or 30 min at 37 °C or 15 °C in

medium or in the presence of BFA. Cells were then washed and re-incubated at 37 °C for 30 min to allow for the internalization of SIINFEKL/Kb complexes formed at the cell surface (especially at 15 °C). Cells were subsequently treated with pronase E (4 mg/ml) at 8 °C for 2 h to clear the cell surface of peptide/MHC-I complexes. After pronase treatment, cells were washed and re-incubated in prewarmed medium at 37 °C for 30 min to allow for the transport of intracellular SIINFEKL/Kb complexes to the cell surface. Cells were finally washed and stained with the anti-Gal2 monoclonal antibody (MC2102) and $F(ab')_2$ FITC-labelled rabbit antimouse antibody.

Intracellular peptide exchange. RMA-S cells (precultured at 26 °C) or iDC were incubated in medium or in the presence of BFA (10 μg/ml) with different concentrations of a Kb-binding inhibitor peptide (RGY8-6h) [30] for 30 min at 37 °C, followed by the addition of the SIINFEKL peptide (30 μg/ml) for another 30 min. Cells were then washed and stained for membrane expression of SIINFEKL/Kb complexes, using the 25-D1 monoclonal antibody. In this way, it was established at which concentrations the RGY8-6h peptide inhibited the binding of the SIINFEKL peptide to Kb. Next, 26 °C cultured RMA-S or iDC (in the presence of BFA) were treated with inhibitory concentrations of the RGY8-6h peptide (30 or 600 μg/ml) for 30 min at 37 °C or 15 °C, followed by the addition of different concentrations of OVA peptide SIINFEKL for another 3 h at respective temperatures. Cells were then washed and stained with the 25-D1 monoclonal antibody.

Results

Endolysosomal MHC I processing in iDC

To investigate OVA processing in iDC and imDC, cells from both wt and $TAP1^{-/-}$ mice were preincubated with a set of inhibitors, including BFA, lactacystin and the lysosomotropic amines ammonium chloride, chloroquine and methylamine. BFA was earlier found to cause a rapid disassembly of the Golgi organelle in DC by using the BODIPY-ceramide labelling of the Golgi system [33], demonstrating that the molecular target for BFA, Sec7GEF, is expressed in these cells [25]. Drug-pretreated cells were incubated with OVA for an additional 4 h, thereafter they were washed and stained with the SIINFEKL/Kb-specific monoclonal antibody 25-D1 [26] (Table 1) and Alex Fluor 647-labelled goat antimouse immunoglobulin. Both untreated and BFA- and lactacystin-treated iDC stained positive for the 25-D1 monoclonal antibody, demonstrating the expression of the SIINFEKL/Kb complex on the surface of these cells (Fig. 1). However, no expression was seen in the presence of any of the lysosomotropic amines used. In addition, no 25-D1 staining was seen on subcultured imDC using the same experimental set-up.

Interestingly, iDC from $TAP1^{-/-}$ mice expressed relatively high levels of complexes in relation to their low Kb expression (approximately 10% of wt), indicating that Kb in these cells is more receptive to the OVA-derived peptide as compared to in wt cells in which most Kb molecules are saturated with endogenous peptides. The same phenomenon was seen, although at a higher level, when cells were treated with the SIINFEKL peptide (data not shown). However, in peptide-treated cells, the expression was proportionally much higher on untreated as compared to BFA-treated cells, especially using $TAP1^{-/-}$ cells, suggesting a rapid efflux of receptive Kb molecules from the ER which eventually became stabilized on the cell surface as SIINFEKL/Kb complexes. Another observation was that lactacystin treatment increased the OVA-derived complex expression on both wt and $TAP1^{-/-}$ DC (Fig. 1) possibly due to an influx of more receptive 'empty' Kb molecules into the processing compartment of drug-treated cells as a consequence of a reduced proteasomal activity.

Internalization and intracellular binding of peptides to Kb

To demonstrate cellular uptake and binding of peptides to Kb, we used low temperature (to induce a higher Kb expression) [34] and BFA-treated RMA-S cells and the SIINFEKL peptide. We treated RMA-S cells with the peptide at 37 or 15 °C. Both of these temperatures allowed fast peptide binding and a similar expression of SIINFEKL/Kb complexes on cells (data not shown), but only cells incubated at 37 °C had the capacity to simultaneously internalize peptides. Peptide-treated cells were then washed, re-incubated at 37 °C to allow the internalization of membrane-bound SIINFEKL/Kb complexes (including those exclusively formed at the membrane level at 15 °C), cleared from membrane expression of Kb and complexes by pronase treatment and again incubated at 37 °C to allow both the potential re-expression of internalized membrane-derived complexes and the expression of complexes formed in the endolysosomes. We reasoned

Table 1 Peptides, glycopeptides and monoclonal antibodies used for the characterization of endolysosomal processing

MHC-I	Peptide/glycopeptide	Designation	Monoclonal antibody	Specificity of monoclonal antibody
Kb	SIINFEKL	SIINFEKL	25-D1	SIINFEKL/Kb complex and undefined membrane epitopes [26]
Kb	SIIh(Gal2)FEKL	SIIN-4h-Gal2	MC2102	Gal2 [27]
Db	ASNENh(Gal2)ETM	ASN9-6h-Gal2	MC2102	Gal2 [27]

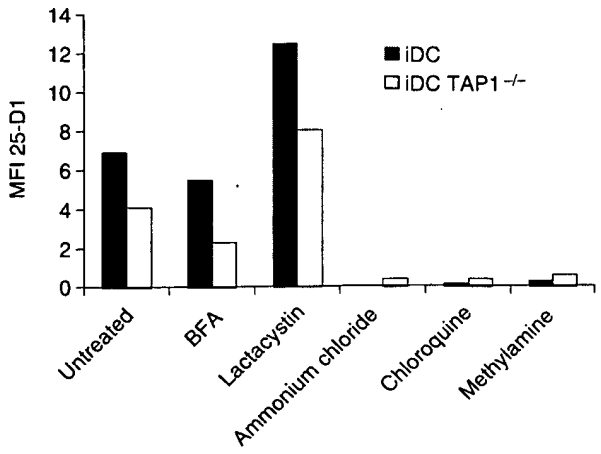

Figure 1 Endolysosomal MHC-I processing in immature DC (iDC). iDC and imDC derived from B6 and TAP1$^{-/-}$ B6 mice were first treated with Brefeldin A (BFA) (10 μg/ml), lactacystin (10 μg/ml), ammonium chloride (20 mM), chloroquine (25 μM) and methylamine (5 mM) for 30 min at 37 °C and then with the OVA protein (10 mg/ml) for an additional 4 h. Cells were washed and stained with the monoclonal antibody 25-D1 followed by a F(ab')$_2$ Alexa Fluor 647-labelled goat antimouse antibody. Mean fluorescence intensity (MFI) is shown after subtraction of the background, cells treated with the secondary antibody only. No staining was seen on imDC (data not shown). One representative experiment of three is shown.

that if such re-appearance would occur only with RMA-S cells that were initially peptide treated at 37 °C, then that would demonstrate peptide uptake and intracellular peptide binding to Kb and exclude the recycling of SIINFEKL/Kb complexes derived from the cell membrane.

However, it turned out that the 25-D1 monoclonal antibody was less useful for this purpose, as it cross-reacted with an undefined membrane epitope expressed on control and pronase-treated RMA-S cells, the latter which were completely devoid of Kb expression. Although the 25-D1 monoclonal antibody has a strict specificity for the SIINFEKL/Kb complex in same situations, it is known to bind to additional, undefined membrane structures under other circumstances [26]. For that reason, we shifted to using Kb or Db binding, synthetic glycopeptides which carried a carbohydrate epitope not expressed in mammalian cells (Gal2, galabiose) coupled by a linker to different positions in the carrier peptide [30] (Table 1). Such glycopeptides could easily be detected when bound to Db and Kb, with high specificity, using the Gal2-specific monoclonal antibody MC2102 [27]. We used both glycosylated forms of the Kb-binding SIINFEKL peptide and the Db-binding ASNENMETM peptide [30]. The SIINFEKL peptide was substituted in position 4 for homocystein to which Gal2 was added through a linker and named SII8-4h-Gal2 and the ASNENMETM correspondingly, ASN9-6h-Gal2. Both had earlier been shown to retain the respective MHC-I binding specificity.

Cells treated for 2 h (Fig. 2A,B) bound more glycopeptide compared to cells treated for 30 min (Fig. 2C,D). It was found that ASN9-6h-Gal2/Db complexes re-appeared only on RMA-S cells, which were initially incubated with the glycopeptide at 37 °C but not on cells incubated at 15 °C (Fig. 2A,B), using the procedure as outlined above. The re-appearance occurred in the presence of BFA and was more complete in RMA-S cells treated for 30 min as compared to cells treated for 2 h (Fig. 2C,D). From these experiments, we conclude that the re-appeared ASN9-6h-Gal2/Db complexes on RMA-S cells most likely were derived from glycopeptide binding to Kb by peptide exchange in endolysosomes and not from complexes formed in the ER or recycled in the cell membrane. The same mechanism was detected in iDC using two different Kb- and Db-binding glycopeptides (Table 1) (Fig. 3). The capacity for peptide uptake, peptide binding to Kb and re-appearance of the peptide/Kb complex on the cells surface was not unique for RMA-S cells and iDC but occurred also in imDC, but not in the DC cell line FSDC [35] (data not shown).

Intracellular peptide exchange in Kb

To verify peptide exchange in Kb in endolysosomes, we treated BFA-blocked RMA-S and iDC first with different concentrations of a Kb-binding inhibitor peptide (RGY8–6 h) and second with the SIINFEKL peptide and stained for the appearance of SIINFEKL/Kb complexes on the cell surface (Fig. 4). By this procedure, we established which RGY8-6h concentrations inhibited SIINFEKL binding to the different cells, cultured in medium alone or in the presence of BFA (Fig. 4A,B). Thereafter, BFA-blocked RMA-S cells and iDC were treated with inhibitory RGY8-6h concentrations and subsequently with the read-out SIINFEKL peptide at 37 or 15 °C for 3 h (Fig. 4C,D). From this experiment, it is clear that Kb molecules can exchange the RGY8-6h peptide for the SIINFEKL peptide at 37 °C, but not at 15 °C, suggesting that peptide exchange occurs in endolysosomes and not at the cell surface.

Discussion

By using monoclonal antibodies to follow OVA processing and intracellular peptide exchange, we have presented additional evidence for a fully functional, most probably endolysosomal MHC-I-processing pathway in iDC in support of our earlier results using the specific B3Z T-cell hybridoma as a functional read-out [24].

Only iDC, and not imDC, were found to have the capacity for alternative processing of exogenous OVA into membrane-expressed SIINFEKL/Kb complexes in line with the finding that only OVA-treated and BFA-blocked iDC activated the B3Z T-cell hybridoma [25]. Using Db- and

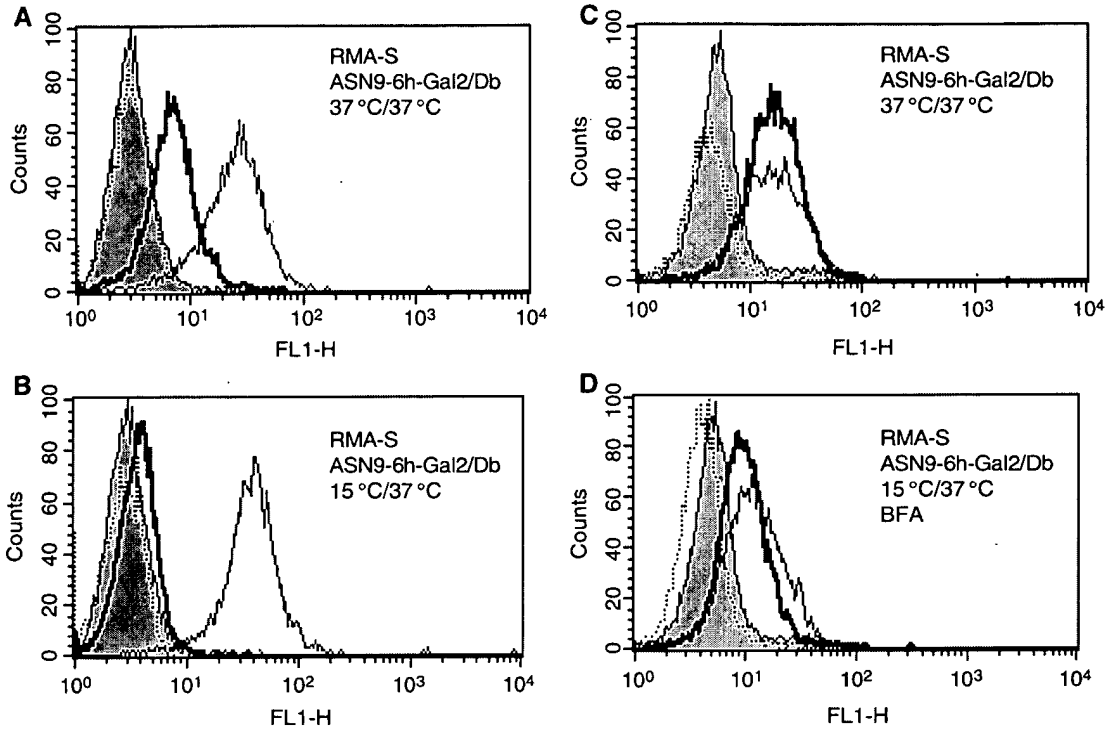

Figure 2 Internalization and intracellular binding of the ASN9-6h-Gal2 glycopeptide to Db and transport of the complex to the cell surface. RMA-S cells (precultured at 26 °C overnight) were treated with the glycopeptide ASN9-6h-Gal2 at either 37 °C (A, light solid line) or 15 °C (B, light solid line) for 2 h. Cells were then washed and re-incubated at 37 °C for 30 min to allow for the internalization of glycopeptide/Db complexes formed at the cell surface (especially at 15 °C). Membrane peptide/Db complexes were then cleared from the cell surface by pronase treatment (A and B, dotted line) at 8 °C for 2 h. Subsequently, cells were further incubated at 37 °C for 30 min and tested for re-expression of the ASN9-6h-gal2/Db complex (A and B, bold solid line). A similar experiment, except that peptide loading was reduced to 30 min instead of 2 h, was done in the presence (D) or absence (C) of Brefeldin A (BFA) (10 μg/ml) with the inhibitor present during the whole assay. Shaded areas represent background staining with the FITC-labelled secondary antibody.

Kb-binding glycopeptides [30] to follow peptide internalization, binding to MHC-I by peptide exchange and the transport of peptide/MHC-I complexes to the cell surface, we found that iDC, imDC and RMA-S cells could perform these functions and that only the DC cell line FSDC [35] scored negative in this respect. Peptide exchange is a well-established mechanism in the MHC-II pathway but has also been reported to occur with MHC-I both in the ER and in low pH endolysosomes, mostly in macrophages [36–38]. Here, we formally show that also myeloid DC can exchange peptides in MHC-I molecules located in a compartment distal to the ER, most probably endolysosomes. The difference between iDC and imDC in terms of alternative MHC-I-processing capacity of exogenous OVA [25] thus does not depend on the later events in this process, peptide binding to MHC-I by peptide exchange and the transport of the peptide/MHC-I complexes to the cell surface. Rather, iDC may be able to perform endolysosomal MHC-I processing, as a consequence of their efficient antigen uptake mechanisms [39, 40] and possibly also because of the presence of certain key proteolytic enzymes in the endolysosomes. We have earlier found that iDC, but not imDC, secreted enzymes into the supernatant that could process OVA into the SIINFEKL peptide and that these were inhibited by AEBSF (serine protease inhibitor), but not by pepstatin (aspartic amino acid inhibitor) and that E64 (cystein protease inhibitor) actually favoured the formation of the SIINFEKL peptide [25]. Endolysosomes contain, in contrast to the cytosole, carboxypeptidases, belonging to the cathepsin family of cystein proteases [41], with the capacity for the unique aspect in MHC-I-processing, cleavage at the C terminus [42].

The number of proteolytic enzymes involved in MHC-I processing is steadily increasing and now comprise enzymes such as TPPII, Furin and several cytosolic proteases as well as endolysosomal enzymes [10, 43–46]. Overall, given the presence of proteolytic enzymes in most cellular compartments, the concept of MHC-guided processing of antigenic peptides as earlier defined for the MHC-II system [47] may sometimes apply also for MHC-I processing. However, as proteolytic fragments generated by proteasomes have structural relationships to MHC-I-binding peptides [48], this enzyme complex has clearly evolved to play a major role in MHC-I processing.

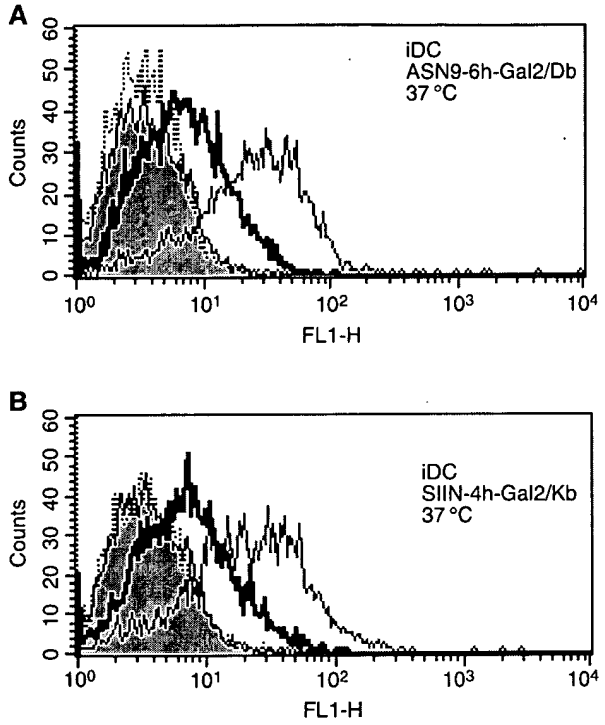

Figure 3 Internalization and intracellular binding of glycopeptides to Db or Kb and transport of the complex to the cell surface in immature DC (iDC). Same procedure as in Fig. 2 using iDC treated with the Db-binding glycopeptide ASN9-6h-Gal2 (A) or the Kb-binding glycopeptide SII8-4h-Gal2 (B).

The classical MHC-I-processing pathway may be especially important for the capacity of infected target cells to signal to CTLs that they contain a foreign protein, i.e. during the effector phase of a cellular immune response. In contrast, during the induction phase of a CTL response, when cross-priming is an important event, other proteolytic enzymes may play a role for the processing of different antigens into MHC-I-binding peptides. Our earlier data showing that an immunostimulatory CpG oligonucleotide could re-activate endolysosomal MHC-I processing in imDC support this idea [25]. The function of endolysosomal MHC-I processing in iDC is unclear but could possibly be important for the generation of tolerogenic DC-internalizing endogenous antigens during their differentiation process.

In summary, we have shown that iDC can process exogenous OVA into a membrane-expressed SIINFEKL/Kb complex in a pathway distinct from the classical MHC-I pathway. This strongly supports the existence of a fully independent endolysosomal MHC-I-processing system. However, the physiological importance of such a system remains to be established.

Acknowledgment

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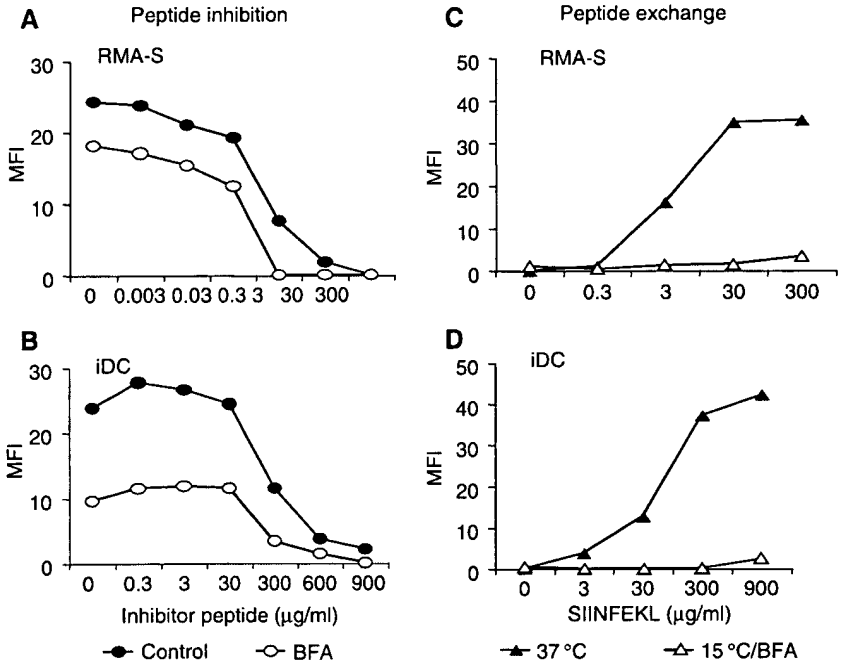

Figure 4 Intracellular peptide exchange in Kb. RMA-S cells (precultured at 26 °C overnight) and immature DC (iDC) were first treated with different concentrations of the RGY8-6h peptide, in medium or in the presence of Brefeldin A (BFA), washed and then treated with the SIINFEKL peptide (30 μg/ml, 30 min) and stained with the 25-D1 monoclonal antibody (A and B), as described in the *Materials and methods* section. To investigate peptide exchange in Kb, cells were saturated with set concentrations of the RGY8-6h peptide (30 μg/ml for RMA-S and 600 μg/ml for iDC) washed and further incubated for 3 h with different doses of the SIINFEKL peptide at 37 or 15 °C in the presence of BFA and finally stained with the 25-D1 monoclonal antibody (C and D).

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